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EFFECT OF PARATHYROID HORMONE-RELATED PROTEIN ON HUMAN PLATELET ACTIVATION

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Running Heading: Parathyroid hormone-related protein and platelet activation

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ABSTRACT

Recent evidence suggests that parathyroid hormone (PTH)-related protein (PTHrP) can act as an inflammatory mediator in several pathological settings including cardiovascular disease. This study was to determine whether PTHrP might be involved in human platelet activation. We used a turbidimetric method to determine platelet aggregation. The expression of the PTH type 1 receptor (PTH1R) in human platelets was analyzed by Western blot and flow cytometry analysis. PTHrP (1-36) (10^{-7} mol L⁻¹) by itself failed to modify the activation of platelets. However, it significantly enhanced ADP-induced platelet activation, and also increased the ability of other agonists (thrombin, collagen and arachidonic acid) to induce platelet aggregation. H89 (10^{-6} mol L⁻¹) and Rp-cAMPS (25×10^{-6} mol L⁻¹), two protein kinase (PK) A inhibitors, and bisindolylmaleimide I (25×10^{-9} mol L⁻¹), a PKC inhibitor, partially decreased the promoting effect of PTHrP (1-36) on ADP-induced platelet activation. Meanwhile, PTHrP (7-34) (10^{-6} mol L⁻¹), a PTH1R antagonist, as well as PD098059 (10^{-5} mol L⁻¹), a mitogen-activated protein kinase (MAPK) inhibitor, or a farnesyltransferase inhibitor abolished this effect of PTHrP (1-36). Moreover, PTHrP (1-36) (10^{-7} mol L⁻¹) increased (2-fold over control) MAPK activation in human platelets. The PTH1R was detected in platelets, and the number of platelets expressing it on their surface in patients during an acute myocardial infarction (AMI) was not different from that in a group of patients with similar cardiovascular risk factors without AMI. Western blot analysis showed that the total PTH1R protein levels were markedly higher in platelets from control than those from AMI patients. PTH1R was found in plasma, where its levels were increased in AMI patients compared with controls. In conclusion, human platelets express the PTH1R. PTHrP can interact with this receptor to enhance human platelet activation induced by several agonists through a MAPK-dependent mechanism.

Key words: PTHrP, human platelets, platelet activation, acute myocardial infarction, mitogen-activated protein kinase.

INTRODUCTION

The acute coronary syndrome is caused by rupture of the atherosclerotic plaque and subsequent occlusive or subocclusive thrombus formation. Activated platelets then release a variety of vasoactive substances that promote platelet aggregation. Increased platelet activation occurs in the setting of cardiovascular disease related to acute coronary syndrome. However, this process is complex and influenced by a myriad of cellular and plasma-derived mediators. In fact, the normal response of platelets can be altered by either an increase or decrease in pro-aggregating or anti-aggregating stimuli, respectively.

Parathyroid hormone (PTH)-related protein (PTHrP), the causative factor of humoral hypercalcemia of malignancy, is now known to be widespread in normal fetal and adult tissues [1]. Due to N-terminal sequence homology between PTH and PTHrP, both peptides interact with a common receptor, the G protein-coupled PTH type 1 receptor (PTH1R), and thus stimulate cAMP formation, protein kinase (PK) C, and mitogen-activated protein kinase (MAPK) activities in different cell types [2-4]. In contrast to PTH, which acts as a classical endocrine hormone, PTHrP normally exerts its effects locally in an autocrine, paracrine, and even intracrine fashion [1,3]. Recent evidence strongly suggests that PTHrP may function as a pro-inflammatory mediator in various pathophysiological settings [5]. Thus, PTHrP may contribute to the lethal effects of several pro-inflammatory cytokines during multi-organ failure in endotoxemia [5-7]. There are also certain evidences supporting the notion that PTHrP, through its pro-inflammatory properties, could be involved in the mechanisms of renal injury [8,9]. Moreover, recent data suggest that PTHrP may also have complex roles in cardiovascular pathology [3]. PTHrP is present in smooth muscle cells and macrophages within human atherosclerotic lesions, where it might act as both pro-inflammatory and pro-atherogenic factor [10,11].

As mentioned above, platelet activation is a key process related to both plaque instability and the compromised blood flow after coronary ischemia [12,13]. To our knowledge, there are no current data on a possible effect of PTHrP on platelet activation, but some investigators have reported some conflicting effects of PTH in platelets. One of these studies has shown that acute administration of physiological concentrations of PTH (1-34) induced an increase in platelet $[Ca^{2+}]_i$ associated with an

increased arterial blood pressure in humans [14]. Moreover, a significant correlation between serum PTH and platelet $[Ca^{2+}]_i$ levels has been found in normotensive patients with primary hyperparathyroidism; although other investigators failed to observe such relationship in human subjects with either essential hypertension or secondary hyperparathyroidism [15,16]. Furthermore, PTH has shown to display either pro- or anti-aggregating effects in human platelets [17,18]. Taken together, however, these results support the notion that PTH might directly interact with platelets; although no attempts have been made to characterize the putative PTH receptor -e.g., the PTH1R or the PTH-specific PTH2 receptor [19]- in platelets. Moreover no studies have yet been done to assess whether PTHrP may also be involved in the regulation of platelet activation. Therefore, in the present study, we investigated: 1) the effect of PTHrP on platelet aggregation, as well as the putative intracellular mechanism involved in this PTHrP action, and 2) the presence of the PTH1R in human platelets, and possible changes in its expression in platelets from patients during an acute coronary syndrome.

MATERIALS AND METHODS

Reagents

Human PTHrP (1-36) was kindly supplied by Drs. A.F. Stewart and A. García Ocaña (Division of Endocrinology and Metabolism, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA). Human PTH (1-34) and human PTHrP (107-139) were obtained from Sigma (St. Louis, MO, USA), and (Asn¹⁰, Leu¹¹, D-Trp¹²) PTHrP (7-34) amide [PTHrP (7-34)] from Bachem (Bubendorf, Switzerland). N-[2-((p-bromocinnamyl)amino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), adenosine 3',5'-cyclic monophosphorothioate Rp-isomer (Rpc-AMPS), bisindolylmaleimide I (BIM), 2'-amino-3'-methoxyflavone (PD098059) and farnesyltransferase (FTase) inhibitor III were obtained from Calbiochem (San Diego, CA, USA). Affinity-purified mouse monoclonal anti-PTH1R antibody was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against phosphorylated extracellular-regulated kinase (ERK) 1/2 (Thr²⁰²/Tyr²⁰⁴) were from Cell Signaling Technology (Beverly, MA, USA). ADP, thrombin, collagen, and arachidonic acid were purchased from Chrono-par Reagents (Havertown, PA, USA).

Platelet-rich plasma (PRP) preparation

PRP was obtained from 31±3 years old healthy volunteers, who were not taken aspirin or any other anti-platelet or anti-inflammatory drugs from at least 15 days before blood extraction. Healthy volunteers signed informed consent. PRP isolation was carried out as previously reported [20]. Briefly, whole blood was obtained in 10% (vol/vol) acid-citrate-dextrose, and centrifuged at 800xg for 15 min, at 20°C. PRP was collected, and platelet number was counted by a Coulter counter. Platelet number was adjusted to 2.5x10⁸ cells ml⁻¹ with platelet-poor plasma obtained from the same subject.

Platelet aggregation

Platelet activation was registered in a lumiaggregometer (Aggrecoorder, four channels; Chrono-Log Corporation, Havertown, Pennsylvania, USA) by the change in light transmission. A platelet-poor plasma sample was used as control for pre-calibration at 100% light transmission. PRP (0.5 ml) was incubated at 37°C for 10 min with continuous stirring in the aggregometer, and was then stimulated with: ADP (10⁻⁶ mol L⁻¹), arachidonic acid (5 x 10⁻⁴ mol L⁻¹), thrombin (0.02 U ml⁻¹), or collagen (2 µg mL⁻¹), in the presence or absence of different PTHrP peptides. In some experiments,

PRP samples were pre-incubated for 10 min with different inhibitors before PTHrP (1-36) and ADP were added. For calculation, only turbidimetry values at 8 min were used; a time period corresponding to the maximal or near-maximal value of the first wave of platelet aggregation [20].

Western blot analysis

Platelets were obtained from PRP by centrifugation at 600xg for 10 min, at room temperature. Isolated platelets were then homogenized in 8 mol L⁻¹ urea, 2% CHAPS w/v, 40 mmol L⁻¹ dithiothreitol, and 0.01% (w/v) bromophenol blue. After centrifugation at 900g, for 15 min at 4°C, the pellet was discarded. Protein (40 µg/lane estimated by bicinchoninic acid reagent (Pierce, Rockford, IL), using bovine serum albumin (BSA) as standard), were separated on 10% polyacrylamide-SDS gels under reducing conditions. Proteins were then blotted onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were then blocked with 5% BSA in phosphate-buffered saline (PBS) with 0.05% Tween-20, by overnight incubation at 4°C. Membranes were then incubated with the anti-PTH1R antibody (sc-12722, Santa Cruz Biotechnology) at 1:1000 dilution, or rabbit polyclonal antibodies against extracellular signal-regulated kinase ERK 1/2 and phosphor pERK 1/2 (Cell Signaling Technology, Beverly, MA, USA) each at 1:1000 dilution, for 1 h at room temperature. An anti-β-actin (Sigma Aldrich) at 1:5000 dilution, was used as a loading control. After extensive washing, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG respectively for 1 h at room temperature and developed by enhanced chemiluminescence (ECL, Amersham). The bands were quantitated by densitometric scanning (ImageQuant, Molecular Dynamics; Sunnyvale, CA). Pre-stained protein markers were used for molecular mass determinations.

Flow cytometry

For flow cytometry studies, blood samples (0.2 ml) were immediately fixed with 1% p-formaldehyde overnight, at 4°C. The blood cells were then washed two times with PBS, and incubated for 30 min at 25°C with the anti-PTH1R antibody (sc-12722 FITC, Santa Cruz Biotechnology). After extensive washing with PBS, the samples were analyzed in a flow cytometer (Beckton Dickinson, San José, CA, USA) as described elsewhere [21]. The number of platelets expressing PTH1R was determined by analysis of a constant number of platelets (5×10^3) for each sample, using forward and side-light

scatter bivariate plots. Non-specific mouse IgG₁ isotype antibody (sc-2855, Santa Cruz Biotechnology) was used as negative control.

Patients

Both platelets and plasma were obtained from six consecutive patients, aged 75 ± 4 years (range 69 to 83), during an acute myocardial infarction (AMI), admitted in the Emergency Unit of Hospital Clínico San Carlos. AMI was diagnosed on the basis of the classic criteria of prolonged chest pain accompanied by serial changes on the standard 12-lead electrocardiogram, as well as raised plasma creatine kinase (CK) (more than twice the upper limit of the normal value measured in the biochemistry laboratory and CK-MB fraction >10% total CK). Two patients presented electrocardiographic ST elevation whereas the other four patients had not ST elevation (non-Q-wave infarction). A criterion for enrollment was admission within 24 hours after the onset of chest pain. A group of 5 patients matched by age, sex and cardiovascular risk factors were enrolled as a control group. None of the patients was taking any anti-inflammatory and anti-thrombotic drug, except aspirin (250 mg) in the AMI group, administered 20-60 min before blood extraction. Patients with history of neoplastic or autoimmune diseases or infections, or any surgical procedure, in the preceding six months were not included. Both control and AMI patients gave informed consent, and the study was approved by the Ethics Committee of Hospital Clínico San Carlos.

Dot-blot in plasma

For dot-blot analysis, 10 µg of total plasma proteins were loaded onto the nitrocellulose membrane in a dot-blot filtration mani-fold apparatus. The amount of proteins was determined by bicinchonimic acid reagent. As described above for Western blot analysis, the membranes were blocked with 5% BSA in PBS with 0,05% Tween-20, and then incubated with the monoclonal antibody against PTH1R (sc-12722, Santa Cruz Biotechnology) at 1:1000 dilution, for 1 h at room temperature. Following incubation with peroxidase-conjugated goat anti-mouse IgG, membranes were developed by ECL. As loading control, a parallel blot containing the same amount of samples was performed and stained with Coomassie.

Statistical analysis

Results are expressed as mean \pm SEM. Statistically significance was assessed by Mann-Whitney test. $P < 0.05$ was considered significant.

RESULTS

Effect of PTHrP (1-36) on platelet activation

Spontaneous platelet activation (% light transmission: $< 8\%$) was not significantly modified by PRP incubation with increasing concentrations of PTHrP (1-36) (% light transmission: 14 ± 3 , corresponding to this peptide at 10^{-7} mol L $^{-1}$). However, PTHrP (1-36) dose-dependently enhanced ADP-induced platelet activation (Figure 1A). Figure 1B shows a representative trace of platelet aggregation triggered by ADP in the presence of different PTHrP concentrations. As mentioned, PTHrP potentiated the maximal platelet aggregation response to ADP but PTHrP did not significantly change the slope of the platelet curve (ADP: 56 ± 4 min; ADP+ 10^{-7} mol L $^{-1}$ PTHrP: 60 ± 6 , pNS). A similar pro-aggregating effect than with PTHrP was also observed with 10^{-7} mol L $^{-1}$ PTH (1-34) (Figure 1C). In contrast, PTHrP (107-139), at 10^{-7} mol L $^{-1}$, which does not seem to signal through the PTH1R [22], failed to modify ADP-induced platelet activation (Figure 1C). Platelet incubation with 10^{-6} mol L $^{-1}$ PTHrP (7-34), a PTH1R antagonist [23], did not by itself modify ADP-induced platelet activation, but blocked the enhancer ability of 10^{-7} mol L $^{-1}$ PTHrP (1-36) on this activation (% inhibition: 89 ± 3). We next tested whether PTHrP (1-36) would also interact with the effect of other known platelet agonists. We found that this peptide PTHrP (1-36), at 10^{-7} mol L $^{-1}$, also increased the ability of thrombin, collagen and arachidonic acid to induce platelet activation (Figure 2).

PTH1R expression in human platelets

Western blot analysis of human platelet extracts revealed a single band of apparent molecular weight 90-kDa [24], consistent with the presence of the PTH1R in these cells (Figure 3). This was further confirmed by flow cytometry analysis, showing that PTH1R immunoreactivity was present on the platelet surface (% platelets expressing the PTH1R: $19.7 \pm 2.2\%$).

Intracellular pathways associated with the effect of PTHrP (1-36) on human platelet activation

Additional studies were carried out to perform an initial characterization of the mechanism involved in platelet activation by PTHrP (1-36). We found that H89 (10^{-6} mol L $^{-1}$) and Rp-cAMPS (25×10^{-6} mol L $^{-1}$), two PKA inhibitors [25,26], and also BIM (25×10^{-9} mol L $^{-1}$), a PKC inhibitor [27], partially decreased the promoting effect of

PTHrP (1-36) on ADP-induced platelet activation (Table 1). None of these inhibitors significantly modified ADP induced platelet activation (Table 1).

PD098059 (10^{-5} mol L⁻¹), a specific inhibitor of the mitogen-activated kinase (MAPK) upstream of ERK 1/2 [28], abolished the effect of PTHrP (1-36) on ADP-induced platelet activation (Figure 4). In the absence of PTHrP, PD098059 (10^{-5} mol L⁻¹) failed to significantly modify ADP-induced platelet activation (Figure 4). Moreover, PTHrP (1-36) (10^{-7} mol L⁻¹) rapidly (≤ 15 min) increased (2-fold vs control) ERK 1/2 phosphorylation in human platelets (Figure 5). No changes in total ERK $\frac{1}{2}$ expression in platelets was observed under the different experimental conditions (data not shown).

ERK activation by G-protein-coupled receptors may occur via Ras [29]. We found here that the FTase inhibitor III (10^{-8} mol L⁻¹), which inhibits protein prenylation and thus Ras activation [30,31], abrogated the effect of PTHrP (1-36) on ERK 1/2 phosphorylation in human platelets (Figure 5), as well as on ADP-induced platelet aggregation. Thus, this aggregation in the presence of this PTHrP peptide (at 10^{-7} mol L⁻¹) plus the FTase inhibitor III was (% light transmission): 62 ± 4 , not different than the corresponding value with ADP alone (% light transmission: 60 ± 3).

PTH1R expression in platelets from patients during an AMI

Table 2 shows the clinical features of both AMI patients and patients with similar cardiovascular risk factors without AMI (control group). The percentage of platelets from patients during AMI expressing PTH1R in their surface was: 17.2 ± 2.8 % which was not different from that observed in platelets from the control group (19.4 ± 2.6 % pNS). Figure 3 shows a representative flow cytometry analysis of PTH1R in platelets from an AMI patient. However, Western blot analysis demonstrated that platelet content of PTH1R were significantly higher in platelets from the control group compared with that observed in the platelets from AMI patients (Figure 6). Moreover, dot-blot analysis demonstrated that plasma obtained from patients during AMI contained higher amounts of circulating PTH1R levels than those in plasma from patients in the control group (Figure 7).

DISCUSSION

PTHrP was initially isolated from hypercalcaemia-associated tumours. However, in the absence of malignancy, PTHrP rather acts as a local growth factor or cytokine in

a broad variety of tissues [1,3]. A growing body of evidence suggests that PTHrP might be an important mediator of the inflammatory host response. In this setting, PTHrP would participate in a network of redundant pathways involving various cytokines and growth factors, e.g., interleukin-6 and angiotensin II, to promote inflammation and fibrosis (Reviewed in 6).

Inflammation is a key process associated with instability of atherosclerotic lesions [32]. Following atheroma rupture, the formation of an occlusive thrombus is the major event related to myocardial infarction and unstable angina. This is accompanied by an inflammatory response which is responsible for most acute coronary syndromes. In fact, increased plasma levels of inflammatory markers have been detected in this scenario, and the degree of inflammation has been associated with these patients' outcome [12,13,33,34].

Studies in human atherosclerotic lesions following atherectomy have shown an intense PTHrP staining in both macrophages and smooth muscle cells which seems to correlate with the severity of the lesion [10,11,35]. Moreover, we recently demonstrated that PTHrP and monocyte chemoattractant factor-1 colocalize in these cells in the atherosclerotic plaque [11]. These findings suggest that PTHrP may act as a pro-inflammatory factor contributing to plaque breakdown and thrombus formation. The factors responsible for PTHrP overexpression in atherosclerosis are unknown. However, vasoactive factors such as thrombin and norepinephrine, two upregulated agents in this condition and well known platelet activators, could induce PTHrP in the plaque neointima [36,37].

In the present study, we examine whether PTHrP might directly interact with platelet activation. PTHrP (1-36) was found to increase significantly the effect of various known platelet aggregation inducers in human platelets. Our findings also indicate that this effect is likely to occur through the PTH1R, based on the following evidences: First, this receptor was detected –for the first time to our knowledge– in human platelets using Western blot and flow cytometry; second, this effect of PTHrP (1-36) was similar to that of PTH (1-34), which bind to the PTH1R with the same affinity [3]; third, it was abolished by PTHrP (7-34), a PTH1R antagonist [23]. Moreover, our results suggest that the N-terminal region of PTHrP is essential to

enhance platelet activation, since PTHrP (107-139), which does not interact with the PTH1R [22], was inefficient in this regard.

The finding that PTHrP (1-36) not only enhanced the effect of ADP but also that of other inducers of platelet activation suggests that this peptide might interact with an ADP-postreceptor mechanism in human platelets. PTH1R activation usually stimulates both cAMP/PKA and $[Ca^{2+}]_i$ /PKC pathways through $G\alpha_s$ and $G\alpha_q$, respectively, in many target cells [3,38]. In the present study, the promoting effect of PTHrP (1-36) on platelet activation was partially inhibited by either PKA or PKC inhibitors. This suggests that both signal transduction pathways can act independently as mediators of this effect of PTHrP (1-36) in human platelets. Interaction with the PTH1R may also activate MAPK signaling in a Ras-dependent or -independent manner in different cell types [2,4,39]. Our present findings suggest the existence of a cross-talk between PTHrP (1-36) activation of PKA and PKC and MAPK pathways in human platelets, since the enhancing effect of this peptide on ADP-inducing platelet activation was abrogated by a MAPK inhibitor. It is noteworthy that in the absence of PTHrP (1-36), this MAPK inhibitor did not affect ADP-induced platelet activation, in agreement with previous findings using platelet activators [40]. Accordingly with the MAPK pathways involvements of PTHrP-dependent platelet activation, PTHrP (1-36) rapidly activated ERK $\frac{1}{2}$ phosphorylation in human platelets. Present data also support the notion that Ras-dependent ERK activation is a key distal signaling pathway integrating the control of platelet activation by PTHrP (1-36). It is of interest in this regard, that this PTHrP domain has shown to activate several G-protein-coupled pathways apparently converging in ERK activation to modulate osteoblast cell differentiation [2]. The true role of ERK activation on platelet activation is currently controversial [40,41]. However, this activation might induce Ca^{2+} influx [41], and thus be responsible, at least in part, for the pro-aggregating effect of PTHrP in human platelets. This hypothesis awaits further studies.

As mentioned above, PTHrP is abundant in human atherosclerotic plaques [10,11]. Previous data and present results support the hypothesis that, after plaque rupture, human platelets would be exposed to PTHrP, and thus might promote both the inflammatory reaction and platelet activation. The presence of PTH1R in platelets, as demonstrated here, might provide a new significance to the previously reported increase in serum PTH early after acute myocardial ischemia [42]. It is unknown whether the

recently identify PTH-specific receptor, the PTH2 receptor [19], could also be present in platelets. However, present data suggest that, in this pathological setting, both PTH and PTHrP might act alone or in concert through the PTH1R to promote platelet aggregation. In the present study, we failed to find significant alterations in the number of platelets expressing the PTH1R in their surface in AMI patients, compared to that in another group of cardiovascular patients without AMI, matched by age, sex and cardiovascular risk factors, used as controls. However, despite the fact that AMI patients were administered anti-platelet therapy (aspirin) before platelet isolation, the total amount of PTH1R in platelets from these patients were markedly reduced compared with those from control. Therefore, taken together these findings suggest that PTH1R may be also contained into the platelets, probably stored in the platelet granules, and when platelets are activated, as occur during AMI, may rapidly been released into the circulation. In this regard, platelets contain other receptors, such as CD40 ligand and P-selectin, which localize to the platelet surface upon activation, and are rapidly shed into the circulation [43-45]. Therefore, we assessed whether such mechanism might also apply to the PTH1R, which could thus be detected in plasma in AMI patients. Our results reveal, for the first time to our knowledge, the presence of this receptor in plasma. In addition, its plasma levels were significantly increased in plasma during AMI. The source of the circulating PTH1R is presently unknown, however, since it might originate not only from platelets but also from other cells of the ruptured plaque environment. In addition, it did not discard that platelets during AMI may interact with PTHrP in the ruptured atherosclerotic plaques which may favour the activation of the expressed PTH1R on the platelet surface enhancing platelet activation during the acute coronary syndrome. In any case, our finding is intriguing and raises the question of the putative functional role of the PTH1R in AMI as a marker of thrombosis or inflammation. In this regard, it would be interesting to explore in future studies the possible relation of the increase in PTH1R plasma levels with that of other soluble platelet receptor, namely sCD40 ligand and P-selectin [43,46,47], in the setting of cardiovascular disease.

In conclusion, our findings demonstrate for the first time to our knowledge, that human platelets express the PTH1R. In addition, this receptor seems to mediate a pro-aggregating effect of both PTH and PTHrP through a mechanism involving ERK activation. Our findings provide new insights into the mechanisms of platelet activation,

which may lead to the development of new pharmacological strategies for patients with acute coronary syndrome.

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LEGENDS OF THE FIGURES

Figure 1.- Bar graphs showing the effect of increasing concentrations of PTHrP (1-36) (A,B) and that of PTH (1-34) and PTHrP (107-139) (each at 10^{-7} mol L $^{-1}$) (C) on ADP (10^{-6} mol L $^{-1}$)-induced platelet activation. (B) Representative platelet aggregation trace showing the effect of ADP alone and in the presence of increasing concentrations of PTHrP. (C) Bar graph showing the effect ADP+PTH(1-34) and ADP+PTHrP(107-139) (each at 10^{-7} mol L $^{-1}$) on ADP-induced platelet activation. For comparison the effect of ADP+PTHrP (10^{-7} mol L $^{-1}$) was also included. Results are mean \pm SEM of four independent experiments. * $p < 0.05$, with respect to ADP alone.

Figure 2.- Bar graph showing the effect of PTHrP (1-36) (10^{-7} mol L $^{-1}$) on platelet activation induced by thrombin (0.02 U ml $^{-1}$), collagen (2 μ g mL $^{-1}$) and arachidonic acid (AA) (5×10^{-4} mol L $^{-1}$). Results are mean \pm SEM of three independent experiments. * $p < 0.05$, with respect to the corresponding value in the absence of PTHrP (1-36).

Figure 3.- Representative autoradiogram showing the PTH1R protein expression in human platelet extracts from two different normal subjects (V $_1$ and V $_2$) by Western blot (see text for details). At the bottom is shown the flow cytometry analysis to detect PTH1R expression on the platelet surface of platelets obtained from patients during AMI and patients with similar cardiovascular risk factors without AMI. In the middle of the figure is showed the autofluorescence of platelets used as negative control. which was subtracted from the positive value of the samples. In addition, a non-specific mouse IgG $_1$ -isotype antibody was also used as negative control (in the figure first column on the left).

Figure 4.- Bar graph showing the abrogating effect of the MAPK inhibitor PD098059 (10^{-5} mol L $^{-1}$) on the pro-aggregating ability of PTHrP (1-36) (10^{-7} mol L $^{-1}$) in ADP-stimulated human platelets. Results are mean \pm SEM of three independent experiments. * $p < 0.05$, with respect to ADP alone. † $p < 0.05$, with respect to ADP+PTHrP (1-36).

Figure 5.- Representative autoradiogram showing ERK 1/2 activation by PTHrP (1-36) (10^{-7} mol L $^{-1}$) and its blockade by the FTase inhibitor III (10^{-8} mol L $^{-1}$) by Western blot. Protein loading was similar in each well, as assessed by Ponceau S staining (not shown).

Figure 6.- Representative Western blot showing the total content of PTH1R in platelets from patients during AMI and patients in the absence of AMI (control group). Densitometric values are mean \pm SEM. * $p < 0.05$ with respect to the control group.

Figure 7.- Representative dot-blot showing the presence of PTH1R protein in plasma of both groups of patients, during AMI and in the absence of AMI (control group). Protein loading was similar in each well as assessed by Coomassie staining (at the bottom). Corresponding densitometric values as mean \pm SEM are shown. * $p < 0,05$ with respect to the control group.

Figure 1A

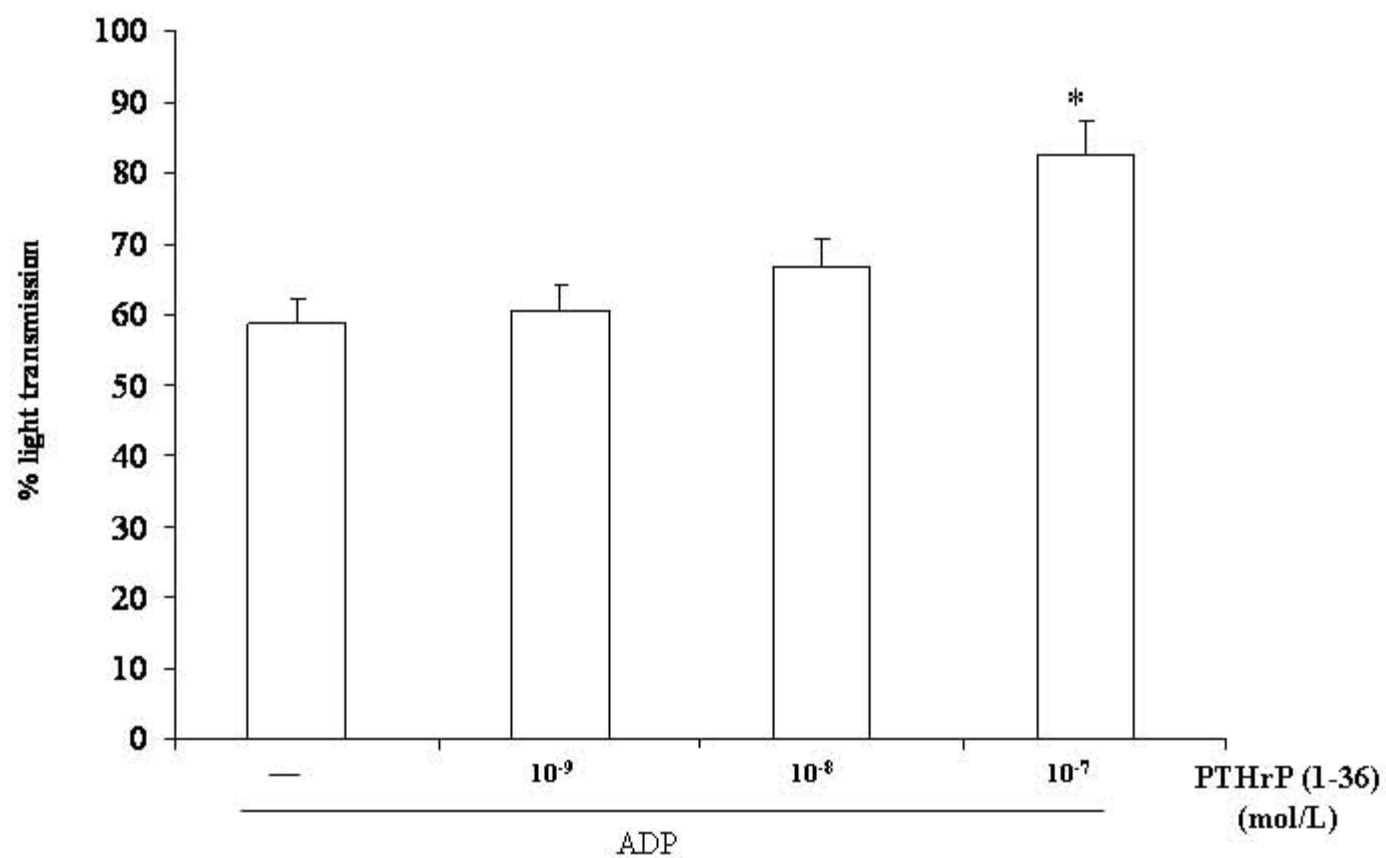


Figure 1B

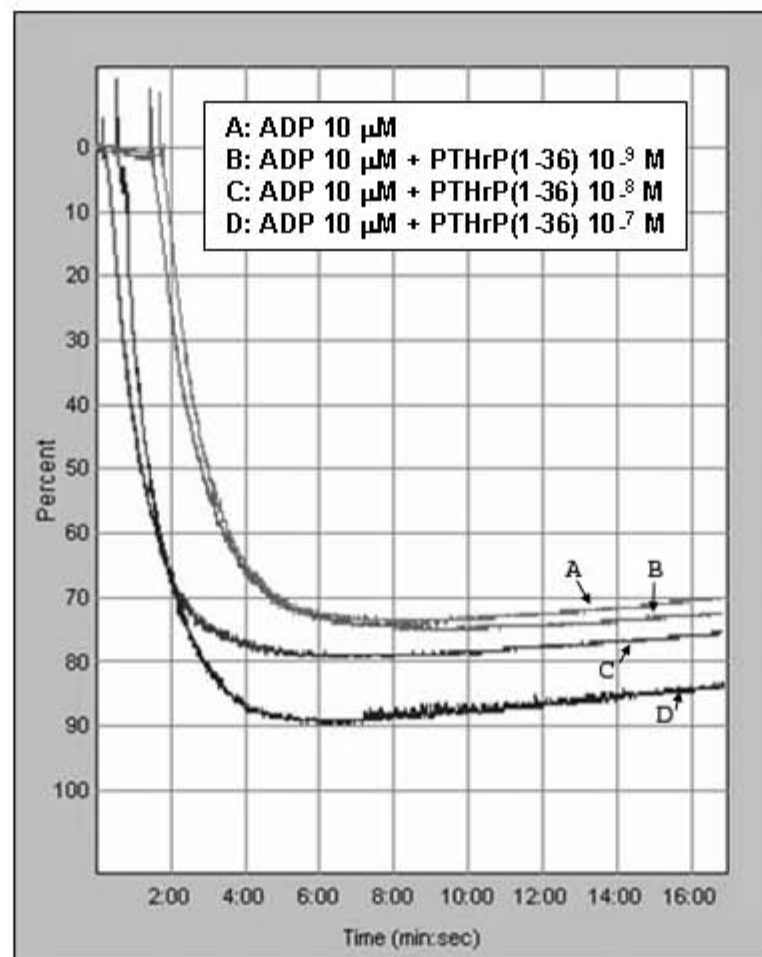


Figure 1C

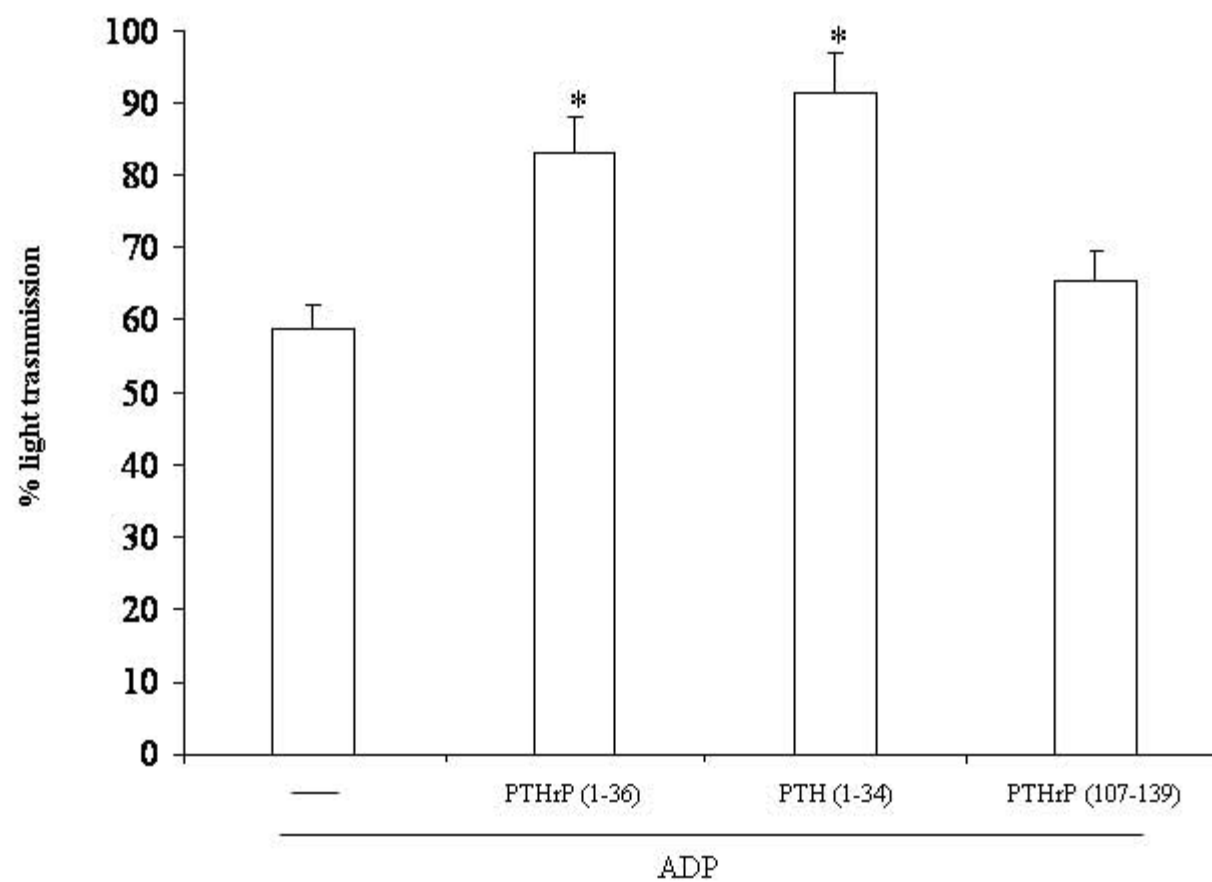


Figure 2

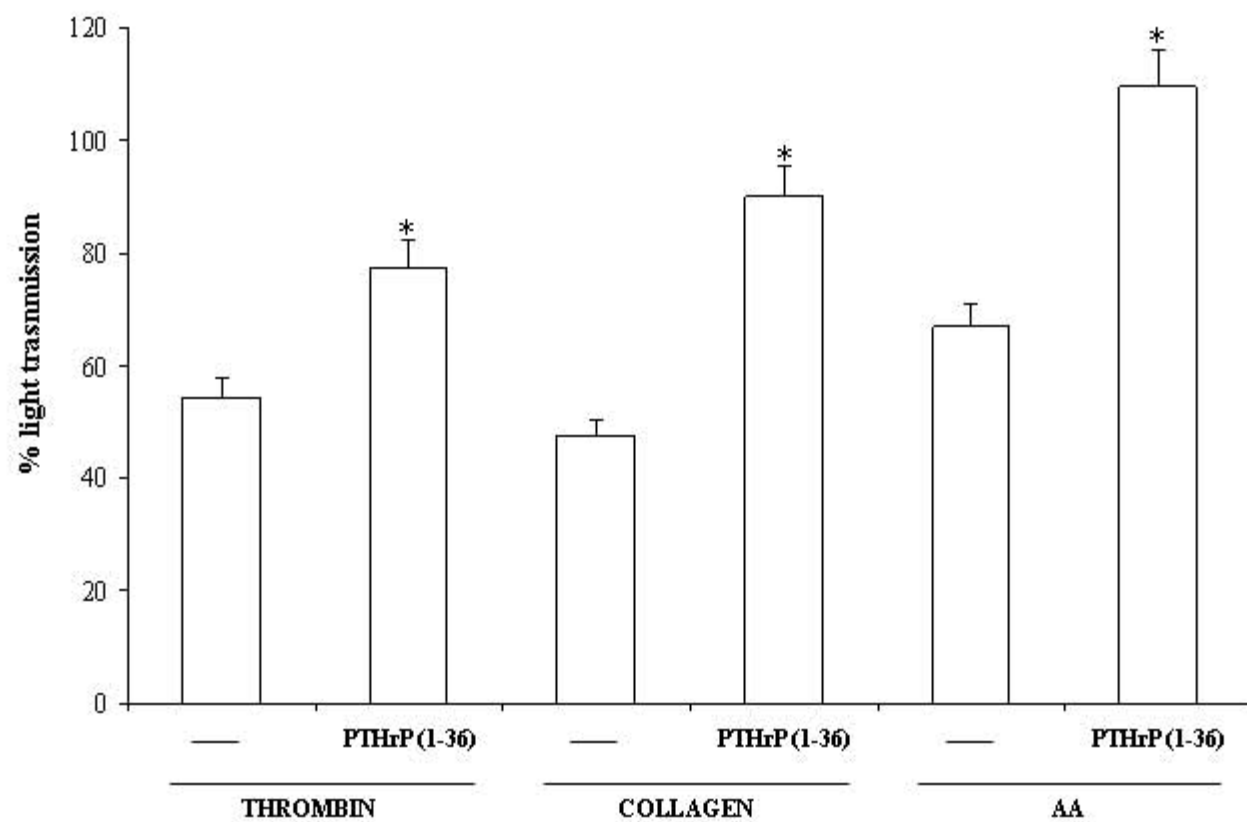
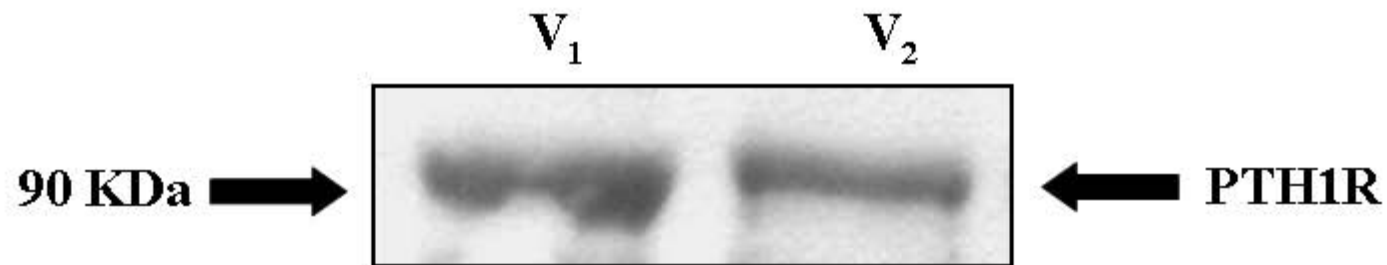


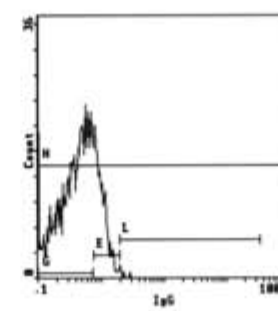
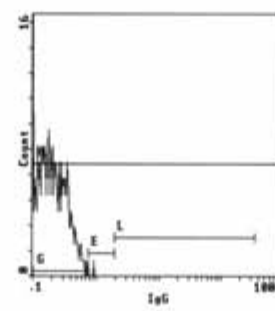
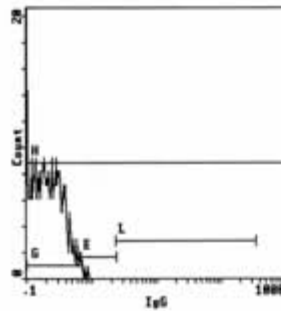
Figure 3



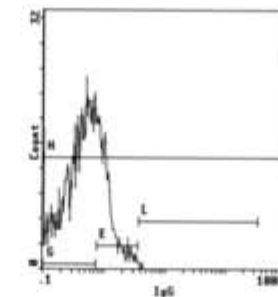
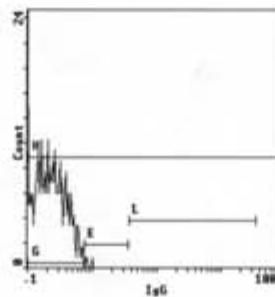
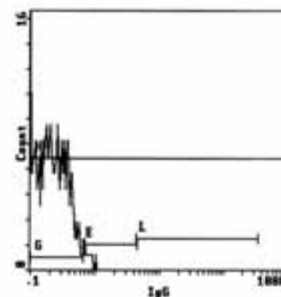
Isotype Control

Autofluorescence

Sample



PTH1R in Control Group



PTH1R in AMI Group

Figure 4

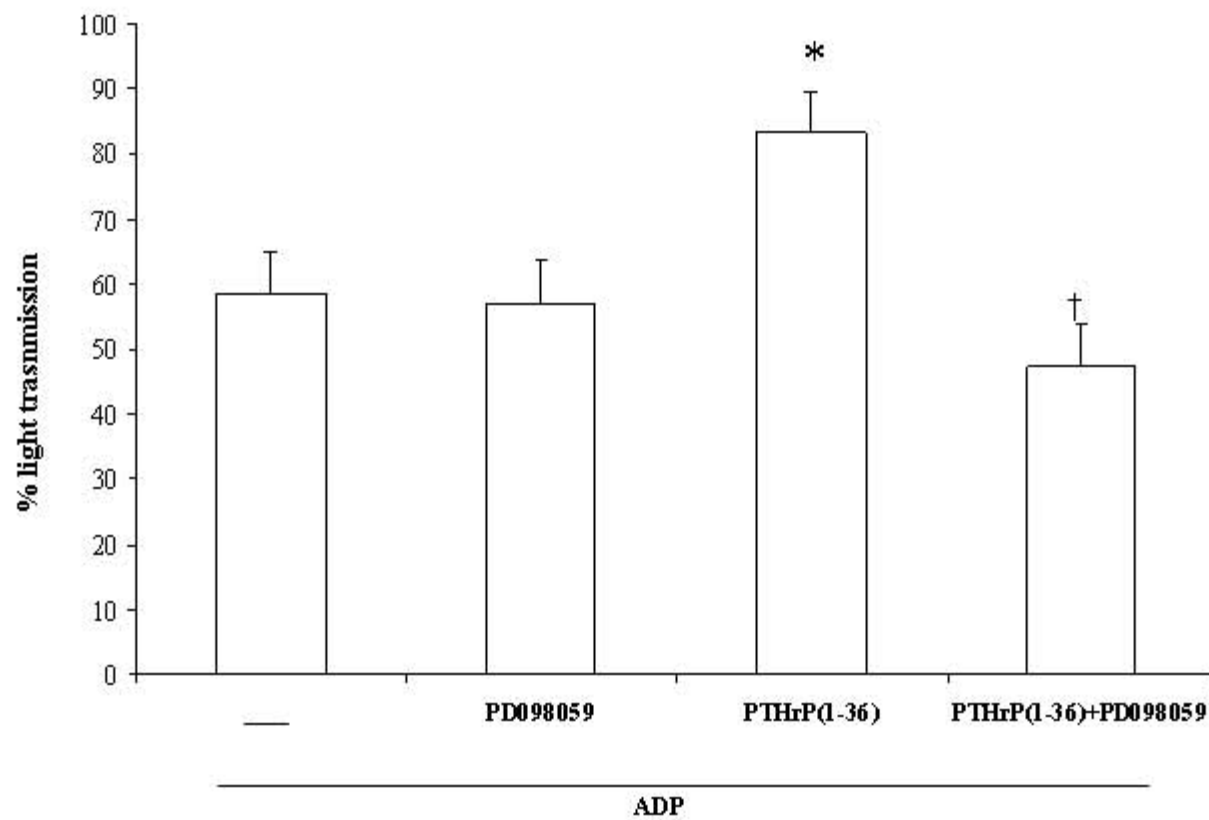


Figure 5

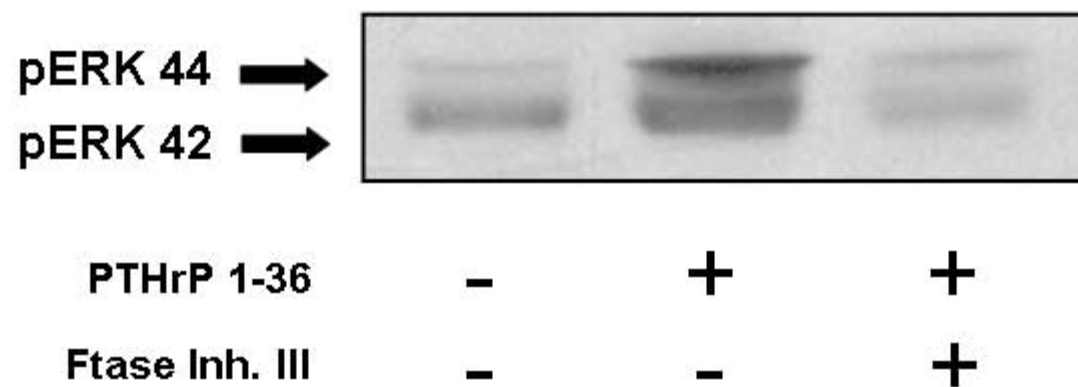


Figure 6

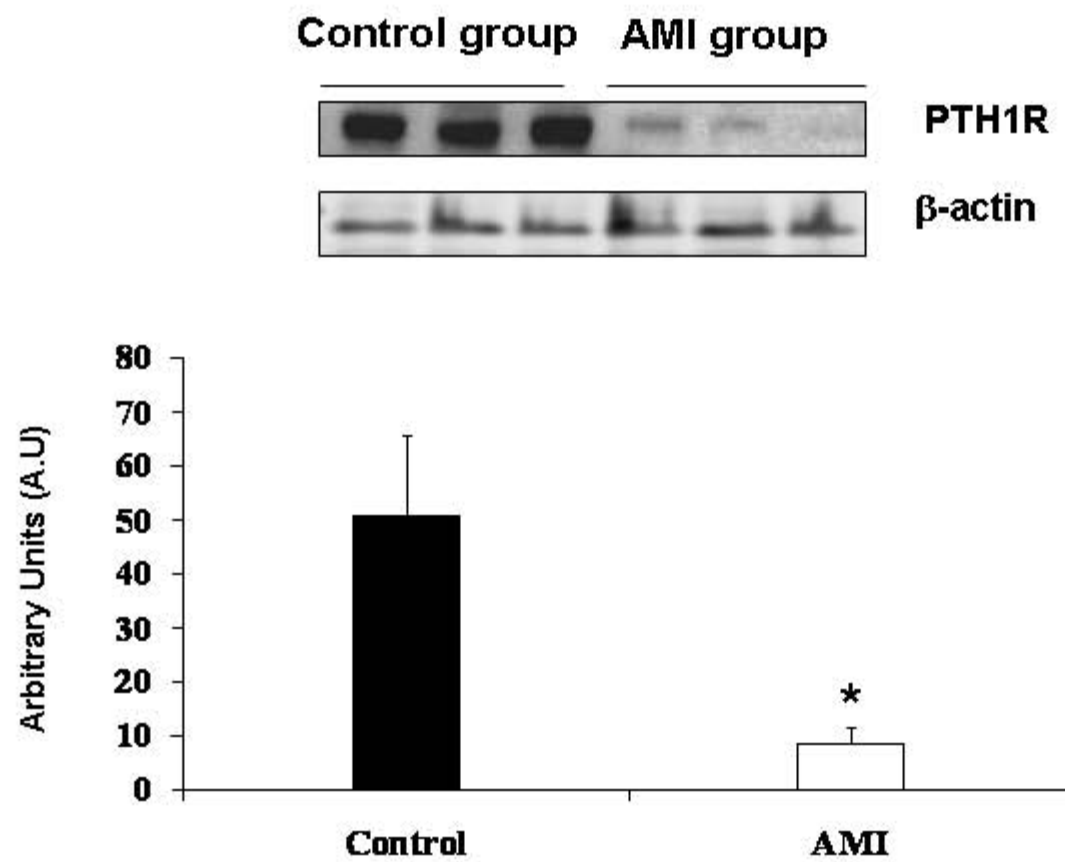


Figure 7

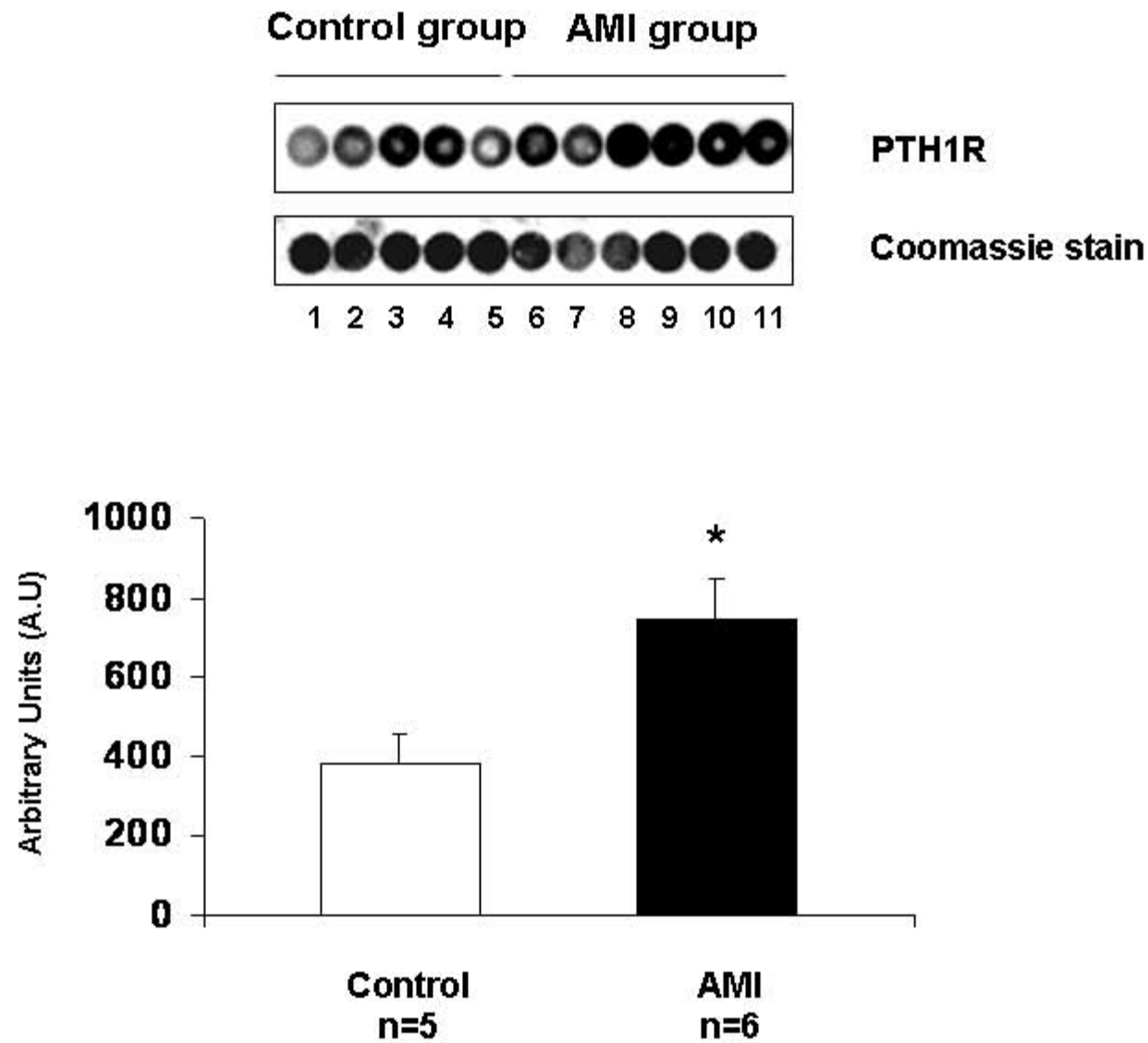


Table 1.- Effect of PKC (BIM) and PKA (H89, Rp-cAMPS) inhibitors on the enhancer ability of PTHrP (1-36) on ADP-induced human platelet activation.

	% Platelet aggregation	
	ADP	ADP + PTHrP (1-36)
-	59 ± 2	83 ± 3*
BIM	53 ± 2	70 ± 2* †
H89	48 ± 3	72 ± 3* †
Rp-cAMPS	58 ± 2	68 ± 2* †

Results are mean ± SEM of four different experiments. * p<0.05, with respect to the corresponding value in the presence of ADP. † p<0.05, with respect to ADP + PTHrP(1-36).

Table 2.- Clinical features of AMI patients and patients with similar cardiovascular risk factors.

	Control group (n = 5)	AMI Group (n = 6)
Age (years)	61,8± 2,26	69 ± 5,1
Male / Female	3 / 2	3 / 3
Hypertension	1 (20%)	3 (50%)
Hypercholesterolemia	5 (100%)	5 (83,3%)
Tobacco smoking	1 (20%)	2 (33,3%)
Diabetes Mellitus	1 (20%)	2 (33,3%)
Drugs		
- ARA II	0 (0%)	1(16,7%)
- Statins	1 (20%)	2 (33,3%)
- β-Blockers	1 (20%)	1 (16,7%)
- Aspirin	0 (0%)	6 (100%)

Abbreviatures: ARAII: Angiotensin II receptor antagonist.